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Hydrogen sulfide inhibits preoptic prostaglandin E_2 production during endotoxemia

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ABSTRACT

Hydrogen sulfide (H_2S) is a gaseous neuromodulator endogenously produced in the brain by the enzyme cystathionine β-synthase (CBS). We tested the hypothesis that H_2S acts within the anteroventral preoptic region of the hypothalamus (AVPO) modulating the production of prostaglandin (PG) E_2 (the proximal mediator of fever) and cyclic AMP (cAMP). To this end, we recorded deep body temperature (Tb) of rats before and after pharmacological modulation of the CBS–H_2S system combined or not with lipopolysaccharide (LPS) exposure, and measured the levels of H_2S, cAMP, and PGE_2 in the AVPO during systemic inflammation. Intracerebroventricular (icv) microinjection of aminooxyacetate (AOA, a CBS inhibitor; 100 pmol) did not affect basal PGE_2 production and Tb, but enhanced LPS-induced PGE_2 production and fever, indicating that endogenous H_2S plays an antipyretic role. In agreement, icv microinjection of a H_2S donor (Na_2S; 260 nmol) reduced the LPS-induced PGE_2 production and fever. Interestingly, we observed that the AVPO levels of H_2S were decreased following the immunoinflammatory challenge. Furthermore, fever was associated with decreased levels of AVPO cAMP and increased levels of AVPO PGE_2. The LPS-induced decreased levels of cAMP were reduced to a lesser extent by the H_2S donor. The LPS-induced PGE_2 production was potentiated by AOA (the CBS inhibitor) and inhibited by the H_2S donor. Our data are consistent with the notion that the gaseous messenger H_2S synthesis is downregulated during endotoxemia favoring PGE_2 synthesis and lowering cAMP levels in the preoptic hypothalamus.

Introduction

Prostaglandin E_2 (PGE_2) is known to play a key role in mediating inflammation. In systemic inflammation, the production of PGE_2 has been reported to occur in peripheral tissues and also in the brain (Steiner et al., 2006). As a result, a number of reactions (namely acute-phase reaction) take place during systemic inflammation, including fever, a brain-mediated response typically generated and controlled in the anteroventral preoptic region of the hypothalamus (AVPO). Fever, a regulated increase in deep body temperature (Tb) that favors the activity of the immune system, is not only the hallmark of infection but also a very important host defense response. Among other microbial products that can trigger fever, lipopolysaccharide (LPS) of the outer membrane of Gram-negative bacteria has been the most used experimental tool to study fever.

Modulators are known to increase (propyretic) or decrease (antipyretic) the febrile response to LPS. Among them, the gaseous neuromodulators nitric oxide (NO) (Steiner et al., 2002a, 2002b) and carbon monoxide (CO) (Steiner and Branco, 2001) acting in the brain have been reported to modulate fever.

Recently, a third gaseous transmitter that regulates neuronal activity has been reported (Kimura et al., 2005). Hydrogen sulfide (H_2S) production is mainly catalyzed in the brain by the enzyme cystathionine β-synthase (CBS) (Abe and Kimura, 1996; Enokido et al., 2005; Lee et al., 2009), whereas in peripheral tissues by cystathionine γ-lyase (CSE) (Yang et al., 2008).

H_2S acts on biological systems via a number of interconnected mechanisms (Wagner et al., 2009). Besides regulating neuronal activity (Kimura et al., 2005), H_2S has been documented to inhibit NFkappaB activity (Lee et al., 2009), affecting PGE_2 production (Li et al., 2009), and stimulate cAMP synthesis (Kimura, 2000; Shao et al., 2011) particularly in the anterior hypothalamus (Kwiatkoski et al., 2012). Considering that PGE_2 is the proximal mediator of fever, and that preoptic cAMP levels profoundly influence the activity of warm-sensitive neurons (Boulant, 1998), modulating fever (Steiner

Abbreviations: 3V, third ventricle; AOA, aminooxyacetate; AVPO, anteroventral preoptic region; cAMP, cyclic adenosine monophosphate; CBS, cystathionine β-synthase; cGMP, cyclic guanosine monophosphate; COX, cyclooxygenase; CSE, cystathionine γ-lyase; icv, intracerebroventricular or intracerebroventricularly; ip, intraperitoneal or intraperitoneally; LPS, lipopolysaccharide; mPGES-1, microsomal prostaglandin E synthase-1; MPO, medial preoptic nucleus; NO, nitric oxide; NOS, nitric oxide synthase; NO_x, nitrite/nitrate; PGE_2, prostaglandin E_2; SE, standard error of the mean; sGC, soluble guanylate cyclase; Tb, deep body temperature.

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and Branco, 2003; Steiner et al., 2002a, 2002b), it seems plausible that H_2S in the AVPO may exert a considerable impact on the febrile response to LPS.

Thus, in the present study we addressed the following key questions: Is H_2S endogenously produced in the AVPO? Does this gas play a thermoregulatory role during LPS fever? And if so, does the H_2S effect depend on AVPO cAMP and/or PGE_2 production?

Materials and methods

Animals

Adult male Wistar rats, obtained from institutional vivarium sources, were group-housed (4 animals per cage) and acclimated for 1 week before experimental use. The rats had free access to water and food and were housed in a temperature-controlled chamber at 24–25 °C (model: ALE 9902001; Alesco Ltda., Monte Mor, SP, Brazil), with a 12:12-h light:dark cycle (lights on at 6:00 AM). All experiments were performed on rats weighing 260–300 g at the time of the experiments, hydrated and fed *ad libitum*. Animal care was carried out in compliance with the guidelines set by the Brazilian College of Animal Experimentation (COBEA), an affiliate of the International Council for Laboratory Animal Science (ICLAS), which included minimizing the number of animals used and their suffering, and had the approval of the Animal Care and Use Committee of the University of São Paulo.

Surgeries

Surgical procedures were performed under ketamine-xylazine anesthesia (100 and 10 mg/kg, respectively; 1 ml/kg, intraperitoneal). Anesthetized animals were fixed (prostrate) on a stereotaxic frame to be implanted with a stainless steel guide cannula (15-mm long, 22-gauge outer diameter) in the third ventricle (3 V) for intracerebroventricular (icv) microinjection, according to the following stereotaxic coordinates (Paxinos and Watson, 2007): anteroposterior: −0.4 mm, lateral: 0.0 mm, and dorsoventral: −7.5 mm from bregma. The guide cannula was fixed to the skull with stainless steel screws and acrylic cement. A tightly fitting stylet was inserted into the guide cannula to maintain patency and prevent infection. Afterwards, a median laparotomy was performed so as to insert a temperature datalogger capsule (SubCue, Calgary, AB, Canada) into the peritoneal cavity to record deep body temperature (Tb). All animals were kept under deep anesthesia throughout the surgical procedures. During the last half of the surgical procedures period, around 10% of the rats responded to algesic stimulus (evoked by a pinch applied to the tail) and then received a supplementary dose of anesthetic. Antibiotic solution (160,000 U/kg benzylpenicilin, 33.3 mg/kg streptomycin, and 33.3 mg/kg dihydrostreptomycin; 1 ml/kg, intramuscular) and analgesic medication (Flunexine; 2.5 mg/kg, 1 ml/kg, subcutaneous) were provided immediately after the surgical procedures. Prior to the experimental procedures, the animals were allowed to recover from the surgical interventions for 5 days.

Drugs

Aminooxyacetate (AOA; inhibitor of the enzyme CBS), sodium sulfide (Na_2S; a donor of H_2S), and bacterial lipopolysaccharide (LPS, serotype 0111: b4) were purchased from Sigma (St. Louis, MO, USA). These drugs were dissolved in pyrogen-free saline.

Microinjection

To perform icv microinjection, we used a microinjection device (model 310, Stoelting, Wood Dale, IL, USA) and a 10-μl syringe (Hamilton, Reno, NV, USA) connected to a microinjection needle (30-gauge outer diameter) with a polyethylene tube (PE 10). Microinjection was performed at a flow rate of 50 nl/min. The microinjection needle, 0.1 mm longer than the guide cannula, was inserted into the guide cannula solely at the moment of the microinjection.

Tb recordings

The animals used to perform the *in vivo* experiments (1, 2, 3 and 4) had their Tb recorded at 5-min intervals for 6 h with the temperature datalogger capsule (SubCue, Calgary, AB, Canada) inserted into the peritoneal cavity.

AVPO sampling

The rats used to assess *ex vivo* the AVPO levels of H_2S, NO_x, cGMP, cAMP and PGE_2 (experiments 5 and 6) were decapitated 2 h after treatments (ip injection and icv microinjection), and their brains were quickly excised and promptly frozen by submersion in dry ice-cold isopentane, and stored at −70 °C. Each brain was serially sectioned in a cryostat from the caudal end so as to reach the AVPO. We then sectioned a 500-μm-thick slice of the hypothalamus, and bilateral samples of the AVPO were excised with a punch needle (2-mm outer diameter).

Assessment of H_2S concentration in the AVPO

H_2S levels were measured as described elsewhere (Francescato et al., 2011; Kwiatkoski et al., 2012; Singh et al., 2009; Stipanuk and Beck, 1982). Briefly, AVPO bilateral samples were homogenized in potassium phosphate buffer (100 mM; pH 7.4) using a microprocessor (VirTis, Gardiner, NY, USA). Each sample (50% w/v; 100 μl) contained L-cysteine (10 mM; 20 μl), pyridoxal 5′-phosphate (2 mM; 20 μl) and PBS (30 μl). The reaction was performed in parafilmed eppendorf tubes and initiated by transferring the tubes from ice to bath at 37 °C. After incubation for 2 h, zinc acetate (1% w/v; 100 μl) was added to trap evolved H_2S followed by tricloroacetic acid (10% w/v; 100 μl) to precipitate proteins and thus stop the reaction. After centrifugation, N,N-dimethyl-p-phenylenediamine sulphate (20 mM; 50 μl) in HCl 7.2 M followed by $FeCl_3$ (30 mM; 50 μl) in HCl 1.2 M was then added to 50 μl of the supernatant, and optical density was measured at 670 nm. The calibration curve of absorbance was obtained using Na_2S solutions (0.1–100 μg/ml). To assess the protein content of the samples, the pellets were diluted in 4 ml of sodium hydroxide (0.1 N). The solution was then assayed by using a protein dye reagent (Bio-Rad Laboratories; Hercules, CA, USA; code number: 500-0006).

Assessment of NO_x concentration in the AVPO

NO_x levels were assessed as previously described (Kwiatkoski et al., 2012; Soriano et al., 2010, 2012). AVPO bilateral samples were homogenized on ice in 100 μl of acetic acid solution (0.1 N) using the microprocessor (VirTis, Gardiner, NY, USA). Briefly, the homogenates were centrifuged at 10,000 *g* for 10 min at 2 °C, and the resultant pellets were later processed to assess the protein content, as briefly described above. Absolute ethanol (60 μl) was then added to 30 μl of the supernatant and, after a 30-min period at 0 °C, the samples were centrifuged at 10,000 *g* for 5 min. The supernatant (5 μl) was injected into a NO analyzer (model 280, Sievers Instruments, Boulder, CO, USA) to assess NO_x concentration by NO–ozone chemiluminescence technique.

Assessment of cGMP concentration in the AVPO

cGMP levels were assessed as previously described (Kwiatkoski et al., 2012; Soriano et al., 2010). AVPO bilateral samples were homogenized on ice using the microprocessor (VirTis, Gardiner, NY, USA) in 100 μl of a 6% (w/v) TCA solution. Briefly, the homogenates

were then centrifuged at 10,000 *g* for 10 min at 2 °C, and the resultant pellets were processed to assess the protein content, as briefly described above. The TCA was extracted with diethyl ether, and the samples were lyophilized. The samples were reconstituted in an assay buffer (2 ml) available in the Amersham Pharmacia Biotech kit (Piscataway, NJ, USA; code number RPN226), and cGMP concentration was assessed by enzyme immunoassay (EIA) according to the manufacturer's instructions.

Assessment of cAMP concentration in the AVPO

cAMP levels were assessed as previously described (Kwiatkoski et al., 2012). AVPO bilateral samples were homogenized on ice in 150 μl of a 6% (w/v) trichloroacetic acid (TCA) solution using the microprocessor (VirTis, Gardiner, NY, USA). Briefly, the resulting homogenates were then centrifuged at 10,000 *g* for 10 min at 2 °C and the pellets processed for protein determination, as briefly described above. The TCA was extracted with water-saturated diethyl ether, and the samples were lyophilized. The samples were then reconstituted in 8 ml of the assay buffer provided in the kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA; code number: RPN225), and cAMP concentration was determined by enzyme immunoassay according to manufacturer's instructions.

Assessment of PGE_2 concentration in the AVPO

PGE_2 levels were assessed as described in our previous reports (Soriano and Branco, 2010; Soriano et al., 2011). AVPO bilateral samples were homogenized (VirTis, Gardiner, NY, USA) on ice in methanol (150 μl) containing indomethacin (1 M). Briefly, the homogenates were centrifuged at 10,000 *g* for 10 min at 2 °C. The resulting supernatants and pellets were used for PGE_2 and protein determination, respectively. The samples were reconstituted in the assay buffer provided in the kit (Cayman, 500141 PGE_2 EIA Kit), and PGE_2 levels were measured using enzyme immunoassay according to the manufacturer's instructions.

Experimental protocols

In all experimental protocols, 24 h before the experimental procedures, the animals were placed in an experiment room with temperature set at 24 °C; this ambient temperature was maintained constant throughout the experiments. Doses of AOA and Na_2S tested in pilot experiments were based on the literature (Kwiatkoski et al., 2012), and those that produced consistent, repeatable responses were adopted in the present study.

Experiments 1–4 aimed at investigating the effects of the pharmacological tools on Tb (in euthermic and febrile rats). In another group of animals, rats were decapitated at the peak of the effervescence phase of the LPS-induced fever, *i.e.*, 2 h after LPS injection, to measure the concentration of H_2S, NO_x, cGMP, cAMP and PGE_2 in the AVPO (Experiments 5–6).

Experiment 1: Effect of icv microinjection of AOA on Tb of euthermic rats

To investigate the putative central effect of CBS inhibition on Tb during euthermia, unanesthetized, freely moving rats received ip injection of saline (1 ml/kg) followed by microinjection of AOA (100 or 300 pmol/2 μl) or saline (2 μl) into the 3 V. Tb was recorded for 7 h, starting 1 h before treatments.

Experiment 2: Effect of icv microinjection of Na_2S on Tb of euthermic rats

We also investigated the putative effect of increased central bioavailability of H_2S on the control of Tb of euthermic rats. Unanesthetized, freely moving rats received ip injection of saline (1 ml/kg) followed by microinjection of Na_2S (260 nmol/2 μl) or saline (2 μl) into the 3 V. Tb was recorded for 7 h, starting 1 h before treatments.

Experiment 3: Effect of icv microinjection of AOA on LPS-induced fever

The putative central effect of CBS inhibition during LPS-induced fever was evaluated in unanesthetized, freely moving rats that received ip injection of LPS (100 μg/kg,1 ml/kg) followed by microinjection of AOA (100 pmol/2 μl) or saline (2 μl) into the 3 V. Tb was recorded for 7 h, starting 1 h before treatments.

Experiment 4: Effect of icv microinjection of Na_2S on LPS-induced fever

To investigate the putative effect of increased central bioavailability of H_2S, unanesthetized, freely moving rats were microinjected into the 3 V with a H_2S donor (Na_2S; 260 nmol/2 μl) or saline (2 μl) immediately after ip injection of LPS (100 μg/kg,1 ml/kg). Tb was recorded for 7 h, starting 1 h before treatments.

Experiment 5: Assessment of the AVPO levels of H_2S during euthermia and LPS-induced fever

Following the *in vivo* approaches, we assessed the levels of H_2S production in the AVPO of euthermic and febrile rats. Unanesthetized, freely moving rats received ip injection of LPS (100 μg/kg,1 ml/kg) or saline (1 ml/kg), and 2 h after the animals were decapitated and the brains processed as described above for the H_2S measurement.

Experiment 6: Effect of icv microinjection of AOA or Na_2S on NO_x, cGMP, cAMP and PGE_2 levels in the AVPO during euthermia and LPS-induced fever

We also measured *ex vivo* in the AVPO of euthermic and febrile rats the putative central effects of (i) inhibition of the CBS or (ii) increased bioavailability of H_2S on the levels of NO_x, cGMP, cAMP and PGE_2. The animals received ip injection of LPS (100 μg/kg,1 ml/kg) or saline (1 ml/kg) followed by microinjection into the 3 V of AOA (100 pmol/2 μl), Na_2S (260 nmol/2 μl), or vehicle (saline, 2 μl). After 2 h, the animals were decapitated and the brains processed as described above for each specific measurement.

Statistical analysis

The values are expressed as mean ± SE. Unpaired *t* test or one-way ANOVA followed by Tukey *post hoc* test was used to assess statistical differences among groups in the *in vivo* experiments (Tb recordings) after area under curve was calculated (thermal/fever index) for each animal's Tb recording, adopting as baseline 36 °C; since thermal/fever index represent area under curve, this index is expressed in °C × min. One-way ANOVA followed by Tukey *post hoc* test was used to assess statistical differences in the other experimental protocols (*ex vivo* measurements). Values of $P<0.05$ were considered statistically significant.

Results

Effect of AOA and Na_2S on Tb of euthermic rats

Before investigating the putative effect of AOA and Na_2S on LPS fever, we performed a set of experiments to evaluate whether the CBS inhibitor and the H_2S donor at a given dose would affect Tb control during euthermia. Intracerebroventricular microinjection of the high dose of AOA evoked a marked, significant increase in Tb not only when compared to the saline-treated group ($P<0.001$; 95% CI: −413.3 to −127.1; saline: 230.3 ± 28.32 °C × min, $n=5$, vs. AOA (high dose): 500.5 ± 27.13 °C × min, $n=4$) but also when compared

to the group treated with a lower dose of AOA ($P<0.01$; 95% CI: −368.1 to −92.59). In contrast, the low dose of AOA did not cause any considerable effect on Tb when compared to the saline-treated group ($P>0.05$; 95% CI: −169.1 to 89.34; AOA (low dose): 270.2 ± 41.59 °C×min, $n=6$) (Figs. 1A and B). As to the Na_2S, icv microinjection of this H_2S donor did not significantly affect Tb when compared to the saline-treated group ($P=0.9701$; 95% CI: −102.5 to 106.1; saline group: 230.3 ± 28.32 °C×min, $n=5$, vs. Na_2S: 228.5 ± 34.72 °C×min, $n=6$) (Figs. 1C and D). The doses of AOA and Na_2S adopted in the present study were based on pilot experiments and a previous report (Kwiatkoski et al., 2012).

Effect of AOA and Na_2S on LPS-induced fever

Since the low dose of AOA and Na_2S did not affect Tb control during euthermia, our next step was to investigate whether at these doses the drugs modulate the febrile response to LPS. Intraperitoneal injection of LPS evoked the typical regulated increase in Tb (the well known LPS-induced fever) (392.6 ± 19.86 °C×min, $n=7$). When the injection of LPS was combined with icv microinjection of AOA, LPS fever was significantly increased ($P<0.05$; 95% CI: −240.9 to −0.5645; saline: 392.6 ± 19.86 °C×min, $n=7$, vs. AOA: 513.3 ± 48.96 °C×min, $n=6$). In the opposite direction, LPS injection combined with icv microinjection of Na_2S (251.2 ± 28.05 °C×min, $n=6$) attenuated fever ($P<0.05$; 95% CI: 21.19 to 261.5, compared to saline) (Figs. 2A and B).

AVPO levels of H_2S

To further explore the involvement of H_2S in LPS fever, besides investigating the effects on fever of central pharmacological modulation of the CBS–H_2S system *in vivo*, we assessed the levels of H_2S endogenously produced in the region of the brain known to be crucial for the generation and control of fever, the AVPO. Basal levels of AVPO H_2S in naïve rats were 0.5870 ± 0.04893 μg/mg protein/h, $n=10$. The animals injected with saline or LPS were decapitated 2 h after the injection for subsequent AVPO sampling. Injection of saline (0.5514 ± 0.06344 μg/mg protein/h, $n=9$) did not significantly alter the AVPO H_2S levels ($P>0.05$; 95% CI: −0.1590 to 0.2301). Differently, LPS induced a pronounced reduction of H_2S production in the AVPO ($P<0.01$; 95% CI: 0.06788 to 0.4732 vs. naïve rats; $P<0.05$; 95% CI: 0.001355 to 0.4686 vs. saline) (Fig. 3).

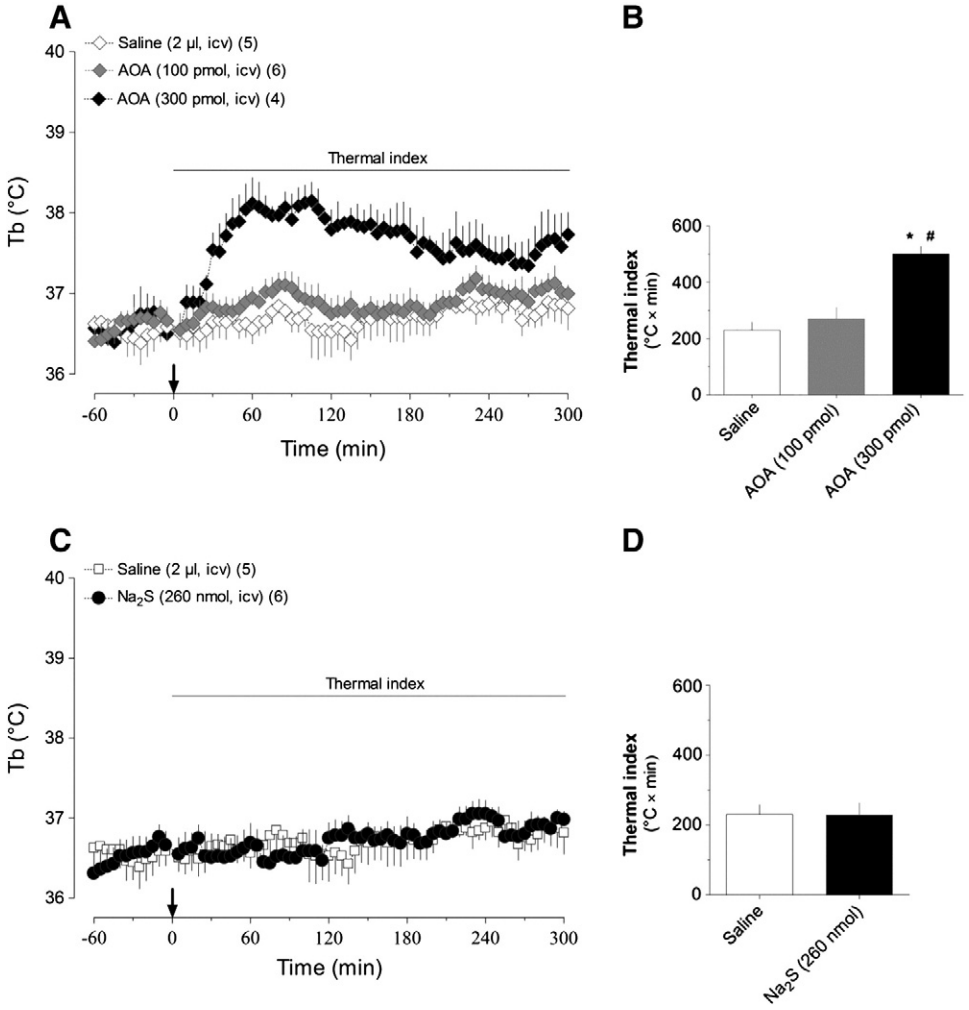

Fig. 1. AOA at 100 pmol does not induce increase and the H_2S donor (Na_2S) at 260 nmol does not evoke decrease in Tb of euthermic rats. A: Time courses showing the effect of icv microinjection of AOA at 100 or 300 pmol in 2 μl or vehicle (saline, 2 μl) on deep body temperature (Tb) of euthermic rats. B: Thermal index (area under curve indicated in panel A by the horizontal bar) clarifies the results observed in the time courses. C: Time courses showing the effect of icv microinjection of Na_2S (260 nmol in 2 μl) or vehicle (saline, 2 μl) on Tb of euthermic rats. D: Thermal index (area under curve indicated in panel C by the horizontal bar) clarifies the results observed in the time courses. Arrow indicates the moment of the microinjection. Values are means ± SE. Number of animals in each group is shown in parenthesis. $^{*}P<0.05$ vs. AOA at 100 pmol, $^{\#}P<0.05$ vs. saline, by one-way ANOVA followed by Tukey *post hoc* test.

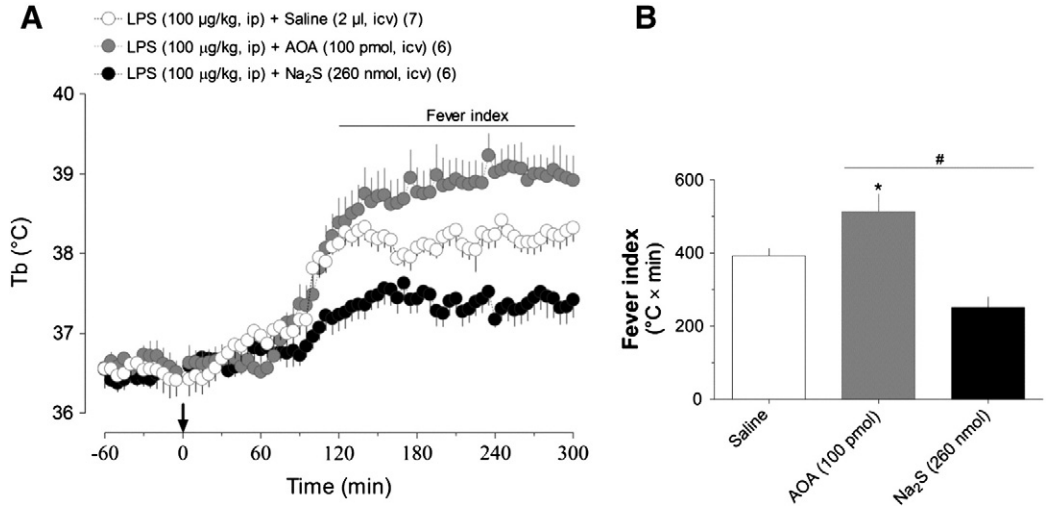

Fig. 2. AOA at 100 pmol exacerbates whereas Na_2S at 260 nmol attenuates LPS fever. A: Time courses showing the effect of icv microinjection of AOA (100 pmol in 2 μl), Na_2S (260 nmol in 2 μl) or vehicle (saline, 2 μl) on fever induced by injection of LPS (100 μg/kg, ip). B: Fever index (area under curve indicated in panel A by the horizontal bar) clarifies the results observed in the time courses. Arrow indicates the moment of the icv microinjection and ip injection. Values are means ± SE. Number of animals in each group is shown in parenthesis. $^{*}P<0.05$ vs. Na_2S, $^{\#}P<0.05$ vs. saline, by one-way ANOVA followed by Tukey *post hoc* test.

AVPO levels of NO_x and cGMP

Since it is known that the levels of both NO and cGMP are reduced in the AVPO during fever, we investigated whether H_2S may affect the AVPO levels of NO and/or cGMP. The animals in this experimental protocol received an icv bolus microinjection of AOA, Na_2S or saline immediately followed by an ip bolus injection of LPS or saline before being decapitated 2 h after these treatments for subsequent AVPO sampling. In the groups of rats treated ip with saline, icv treatment with AOA or Na_2S did not significantly alter ($P>0.05$) the AVPO NO_x levels when compared to the group treated with saline given icv. Similarly, in LPS-treated rats, AOA (4.291 ± 1.055 pmol/μg protein, $n=8$) or Na_2S (5.080 ± 0.8745 pmol/μg protein, $n=8$) did not significantly affect ($P>0.05$) the AVPO NO_x levels when compared to the group treated with saline given icv (3.900 ± 0.5322 pmol/μg protein, $n=7$). Comparing the groups treated ip with saline to the groups treated ip with LPS, we observed that the ip injection of LPS, in all three groups of rats, induced a reduction of the AVPO NO_x levels; such reduction was statistically significant when the group treated with LPS ip combined with saline icv was compared to the group treated with saline ip combined with saline given icv ($P<0.05$; 95% CI: 0.05846 to 7.562; saline ip + saline icv: 7.710 ± 0.6034 pmol/μg protein, $n=8$, vs. LPS ip + saline icv: 3.900 ± 0.5322 pmol/μg protein, $n=7$) (Fig. 4A). As to the levels of cGMP in the AVPO, in the groups of rats treated ip with saline, icv treatment with AOA or Na_2S did not significantly alter ($P>0.05$) the AVPO cGMP levels when compared to the group treated icv with saline (1.629 ± 0.1272 fmol/μg protein, $n=8$). Similarly, in LPS-treated rats, AOA (0.8525 ± 0.1129 fmol/μg protein, $n=8$) or Na_2S (0.5013 ± 0.1172 fmol/μg protein, $n=8$) did not significantly affect ($P>0.05$) the AVPO cGMP levels when compared to the group treated icv with saline (0.5171 ± 0.1020 fmol/μg protein, $n=7$). When comparing the groups treated ip with saline to the groups treated ip with LPS, we observed that LPS evoked a significant reduction of AVPO cGMP ($P<0.01$; 95% CI: 0.3095 to 1.914, comparing saline ip + saline icv with LPS ip + saline icv) (Fig. 4B).

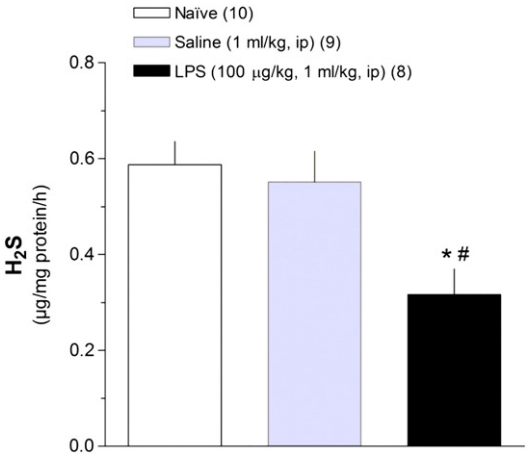

Fig. 3. H_2S levels are reduced in the AVPO during LPS fever. Basal levels and effect of injection of LPS (100 μg/kg, ip) or saline (1 ml/kg, ip) on the AVPO levels of H_2S. Values are means ± SE. Number of animals in each group is shown in parenthesis. $^{*}P<0.05$ vs. saline, $^{\#}P<0.05$ vs. naïve, by one-way ANOVA followed by Tukey *post hoc* test.

AVPO levels of cAMP

Besides cGMP, it is also known that cAMP levels in the AVPO are reduced during fever. Therefore, we then explored the possibility that H_2S is able to modulate adenylate cyclase activity in this hypothalamic region following LPS exposure, *i.e.*, during the febrile response to LPS. The animals in this experimental protocol received an icv bolus microinjection of AOA, Na_2S or saline immediately followed by an ip bolus injection of LPS or saline before being decapitated 2 h after these treatments for subsequent AVPO sampling. Basal levels of cAMP were 16.08 ± 0.9281 fmol/μg protein, $n=8$. In the groups of rats treated ip with saline, icv treatment with AOA or Na_2S did not significantly alter ($P>0.05$) the AVPO cAMP levels when compared to the group treated icv with saline. LPS treatment significantly reduced ($P<0.05$) the AVPO cAMP levels in all three groups investigated when compared to the saline-treated groups ($P<0.001$; 95% CI: 4.926 to 13.14, comparing saline ip + saline icv: 16.08 ± 0.9281 fmol/μg protein, $n=8$, with LPS ip + saline icv: 7.043 ± 0.4325 fmol/μg protein, $n=7$). Despite not statistically significant, when compared with the group treated with LPS (ip) combined with saline (icv), AOA tended to exacerbate the reduction of the AVPO levels of cAMP ($P>0.05$; 95% CI: −3.213 to 4.999). Interestingly, in the group of rats treated with LPS (ip) combined with the H_2S donor (icv) (11.50 ± 0.6053 fmol/μg protein, $n=8$) the LPS-induced reduction of the AVPO cAMP levels was less pronounced ($P<0.05$; 95% CI: −8.563 to −0.3509, compared with

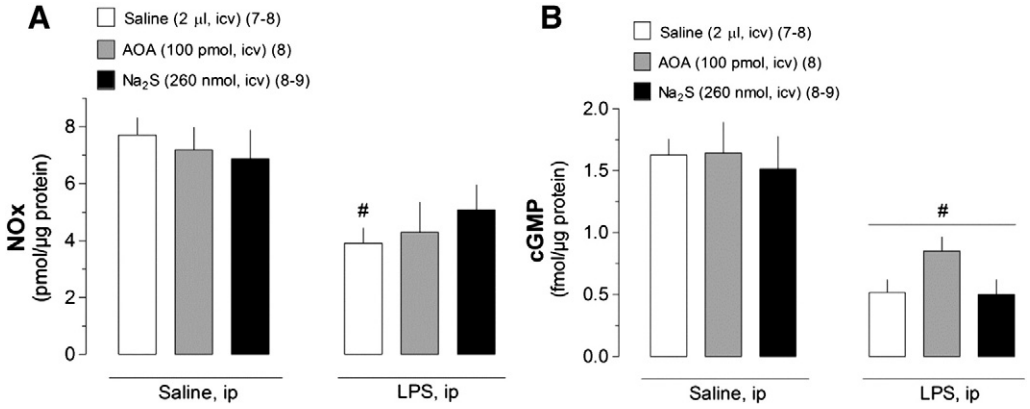

Fig. 4. Neither AOA nor Na_2S affects the AVPO levels of NO_x and cGMP. A: Effect of injection of LPS (100 µg/kg, ip) or saline (1 ml/kg, ip) combined with icv microinjection of AOA (100 pmol in 2 µl), Na_2S (260 nmol in 2 µl) or vehicle (saline, 2 µl) on AVPO nitrite/nitrate (NO_x) levels. B: Effect of the same treatments (described in panel A) on AVPO cGMP levels. Values are means ± SE. Number of animals in each group is shown in parenthesis. $^{\#}P<0.05$ vs. groups treated ip with saline, by one-way ANOVA followed by Tukey *post hoc* test.

LPS ip + saline icv; and $P<0.01$; 95% CI: −9.317 to −1.383, compared with LPS ip + AOA icv: 6.150 ± 0.5904 fmol/µg protein, $n=8$) (Fig. 5).

AVPO levels of PGE_2

Considering the fact that the AVPO levels of the proximal mediator of fever, PGE_2, are known to be increased following LPS challenge, we investigated whether H_2S may modulate the production of this prostanoid. The animals in this experimental protocol received an icv bolus microinjection of AOA, Na_2S or saline immediately followed by an ip bolus injection of LPS or saline before being decapitated 2 h after these treatments for subsequent AVPO sampling. As expected, treatment with LPS evoked a significant ($P<0.05$) elevation in the AVPO PGE_2 levels when compared to the saline-treated groups. In the groups injected ip with saline, neither AOA nor Na_2S significantly affected PGE_2 production ($P>0.05$). As to the effects of these drugs during fever, *i.e.*, in the LPS-treated groups, AOA further increased ($P<0.05$; 95% CI: −117.0 to −1.705; from 185.6 ± 19.27 ng/mg protein, $n=7$, to 244.9 ± 15.78 ng/mg protein, $n=8$), whereas Na_2S significantly reduced ($P<0.05$; 95% CI: 6.922 to 122.2) the AVPO levels of PGE_2 (from 185.6 ± 19.27 ng/mg protein, $n=7$, to 121.0 ± 20.76 ng/mg protein, $n=8$) (Fig. 6).

Discussion

The findings of the present study are consistent with the notion that LPS exposure reduces the AVPO levels of H_2S besides increasing the activity of AVPO COX-2/microsomal PGE synthase-1 (mPGES-1), which is known to ultimately result in the febrile response to LPS. Since we demonstrated that pharmacological inhibition of CBS in rats challenged with LPS (i) further increased the AVPO levels of the proximal mediator of fever, PGE_2, (ii) induced an exaggerated fever, and (ii) tended to exacerbate the reduction of AVPO cAMP, we conjecture that the LPS-induced AVPO reduced levels of H_2S diminish the suppression by H_2S of the AVPO levels of PGE_2 thus favoring the occurrence of the typical regulated increase in Tb (fever). Together with the results of the other experimental protocols of the present study in which the H_2S donor was used, these exciting findings with the CBS inhibitor provide solid evidence not only in vivo but also ex vivo that allow us to suggest that H_2S is a powerful endogenous antipyretic molecule in the preoptic hypothalamus.

In the central nervous system, H_2S is produced by the enzyme CBS, which has been demonstrated to be expressed mainly in astrocytes (Chen et al., 2004; Enokido et al., 2005; Lee et al., 2009). As reviewed by Kimura et al. (2012), reciprocal interaction exists between neurons and astrocytes: surrounding astrocytes are activated by neuronal

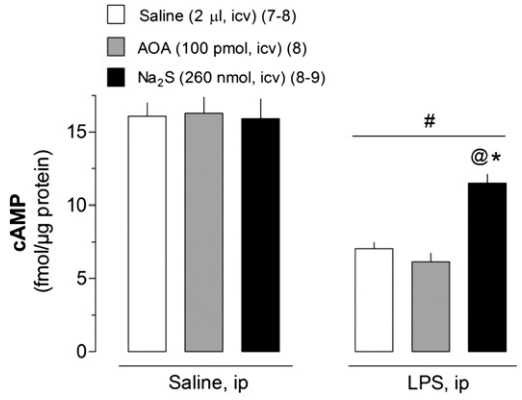

Fig. 5. Na_2S stimulates AVPO cAMP production during fever. Effect of injection of LPS (100 µg/kg, ip) or saline (1 ml/kg, ip) combined with icv microinjection of AOA (100 pmol in 2 µl), Na_2S (260 nmol in 2 µl) or vehicle (saline, 2 µl) on AVPO cAMP levels. Values are means ± SE. Number of animals in each group is shown in parenthesis. $^{*}P<0.05$ vs. AOA + LPS, $^{@}P<0.05$ vs. saline + LPS, $^{\#}P<0.05$ vs. groups treated ip with saline, by one-way ANOVA followed by Tukey *post hoc* test.

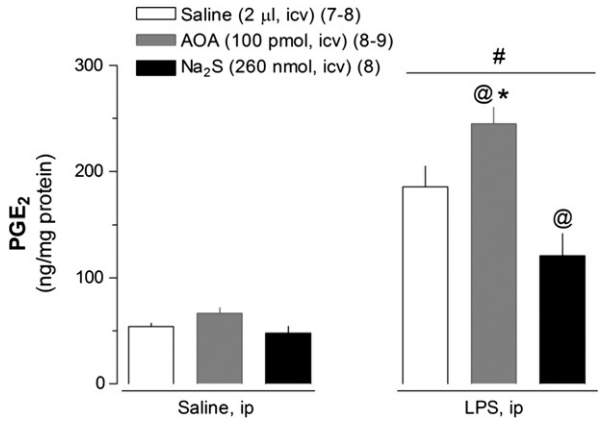

Fig. 6. AOA induces increase whereas Na_2S evokes decrease in the AVPO PGE_2 production during fever. Effect of injection of LPS (100 µg/kg, ip) or saline (1 ml/kg, ip) combined with icv microinjection of AOA (100 pmol in 2 µl), Na_2S (260 nmol in 2 µl) or vehicle (saline, 2 µl) on AVPO PGE_2 levels. Values are means ± SE. Number of animals in each group is shown in parenthesis. $^{*}P<0.05$ vs. Na_2S + LPS, $^{@}P<0.05$ vs. saline + LPS, $^{\#}P<0.05$ vs. groups treated ip with saline, by one-way ANOVA followed by Tukey *post hoc* test.

excitation and in turn modulate synaptic activity. In such interaction, H_2S acts as a regulator of synaptic activity by enhancing the activity of both neurons and astrocytes, and induces Ca^{2+} influx in astrocytes that propagates to the surrounding astrocytes (Enokido et al., 2005; Nagai et al., 2004). Expectedly, reciprocal interactions also take place between astrocytes and neurons in the AVPO, accounting for the generation and control of fever (Blatteis, 2006; Feleder et al., 2004). In this context, AVPO astrocytes stand for a common site of production of both H_2S from CBS and PGE_2 from COX-2/mPGES-1. Therefore, these cells are most likely to be the brain cells mainly affected by the treatment with AOA, and to influence the warm-sensitive neurons activity through PGE_2 release (for a review see Blatteis, 2006).

Considering H_2S as an antipyretic molecule implies that this gas does not affect Tb under normal conditions (euthermia) but is effective in attenuating the regulated increase in Tb (fever). Indeed, our findings showed that central pharmacological inhibition of H_2S production with AOA (100 pmol) did not affect Tb of euthermic rats whereas potentiated the febrile response in rats challenged with a polyphasic fever-inducing dose of LPS (100 μg/kg, ip). In agreement with the role of H_2S in blunting fever, increased bioavailability of this gas in the cerebroventricular system did blunt LPS fever, whereas had no significant effect on Tb.

To our knowledge, the present study is the first to explore in vivo and ex vivo the role of the CBS–H_2S system in the most manifest and familiar sign of the acute-phase reaction of systemic inflammation. Interestingly, here we demonstrated not only that H_2S acts in vivo as an antipyretic molecule in the brain but also that LPS fever is accompanied by reduced levels of H_2S in the hypothalamic region essentially involved in the generation and control of fever, the AVPO. The latter finding is in agreement with the in vitro study by Lee et al. (2009) who showed that H_2S production is suppressed by inflammatory stimulation of astrocytes. The systemic inflammation-associated suppression of AVPO H_2S production (Fig. 3) is likely to reduce the suppressive effect of the gas on the AVPO PGE_2 production, thereby permitting the LPS-induced increase in the levels of PGE_2 in this fever-generating hypothalamic region (Fig. 6).

As reported by Boulant (1998) and reviewed by Steiner and Branco (2003), both cAMP and cGMP have a profound impact on the activity of warm-sensitive neurons of the preoptic hypothalamus, triggering febrile response when their levels are reduced in the AVPO following LPS exposure (Steiner et al., 2002a, 2002b). Consistent with this fact, our findings showed that the levels of cAMP and cGMP were reduced in the AVPO of LPS-treated rats. However, as to the putative effect of H_2S on the NOS–NO–sGC–cGMP system, our findings revealed that, in spite of the fact that interactions have been reported to exist between NO and H_2S (Oh et al., 2006; Whiteman and Moore, 2009; for a review see Kajimura et al., 2010), in the AVPO interactions between these gaseous modulators do not seem to occur, as in this hypothalamic region the treatment with AOA or Na_2S did not affect the levels of NO_x and cGMP either during euthermia or during fever.

The scenario seems different from that of cGMP when we look at the effects of H_2S on the AVPO levels of cAMP and PGE_2. Increased bioavailability of H_2S, besides attenuating fever, remarkably reduced the AVPO PGE_2 levels following the immunoinflammatory challenge. These effects clearly demonstrate the antipyretic capacity of H_2S, confirmed by the attenuated fever (Figs. 2A and B), which adds to the existent data regarding the central antiinflammatory action of the gas in vitro (Lee et al., 2009). Moreover, concomitant with the LPS exposure, the suppression of endogenous H_2S synthesis with AOA further enhanced the PGE_2 production in the AVPO and exacerbated the febrile response, providing again solid evidence of the antipyretic role of endogenous H_2S. This inhibitory effect of the gas on PGE_2 synthesis has been postulated to occur through inhibition of NFkappaB activation (Lee et al., 2009). This possibility may be greatly strengthened by the fact that (i) LPS strongly induces COX-2 and mPGES-1 through the MyD88-dependent NFκB pathway in cultured astrocytes (Font-Nieves et al., 2012), and (ii) the LPS-induced activation of NFkappaB has been shown to be suppressed by H_2S, leading to an inhibition of COX-2 expression (Li et al., 2009; Oh et al., 2006).

As discussed here, induction of systemic inflammation with LPS reduced the AVPO levels of H_2S, and the combined treatment with LPS and AOA revealed that H_2S suppresses PGE_2 synthesis in the AVPO during fever, but not during euthermia. Additionally, our data showed that the gas stimulates cAMP production, but only following LPS exposure, at least in the AVPO. This H_2S-mediated increased synthesis of cAMP has been also shown in our previous report (Kwiatkoski et al., 2012) and by others (Kimura, 2000; Njie-Mbye et al., 2012; Shao et al., 2011). It has been proposed that such stimulatory effect of the gas on the activity of adenylate cyclase is mediated by K_{ATP} channels (Njie-Mbye et al., 2012), and interestingly, in a positive feedback regulatory loop, the adenylate cyclase by-product, cAMP, has been reported to increase the expression of CBS in astrocytes (Kimura et al., 2012). Moreover, in addition to the above mentioned inhibition mediated by H_2S, it is also possible that cAMP itself may inhibit COX-2 expression (Tazawa et al., 1994; Wu et al., 2008), which would characterize the existence of reciprocal interactions between the COX-2/mPGES-1/PGE_2 and adenylate cyclase/cAMP systems, as PGE_2 is known to inhibit adenylate cyclase activity (see below). This cAMP-mediated inhibition of COX-2 would potentially be a mechanism acting concomitantly with that of H_2S-mediated NFkappaB inhibition to reduce COX-2 activity.

Typically, in preoptic neurons PGE_2 activates EP3 receptors to ultimately produce fever (for a review see Morrison and Nakamura, 2011). Once activated, EP3 receptors inhibits adenylate cyclase activity through protein Gi/o, reducing the intracellular levels of cAMP (Bilak et al., 2004; Steiner and Branco, 2002; Toh et al., 1995). Reduced levels of cAMP inhibit the activity of warm-sensitive neurons of the preoptic hypothalamus (Boulant, 1998; for a review see Steiner and Branco, 2003), thereby releasing the tonic inhibitory (GABAergic) inputs onto rostral medullary raphe, generating fever (for a review see Morrison and Nakamura, 2011).

Thus, alternatively or concomitantly to a greater inhibition of PGE_2 production, increased levels of AVPO H_2S can stimulate cAMP production in the AVPO, thereby ultimately serving to prevent an exaggerated fever from occurring during systemic infectious challenges.

Conclusions

Our data are consistent with the notion that the gaseous messenger H_2S is a powerful endogenous antipyretic molecule that may act through suppression of PGE_2 synthesis and/or stimulation of cAMP production in the preoptic hypothalamus (Fig. 7).

Conflict of interest

The authors declare that there is no conflict of interest.

Role of the funding sources

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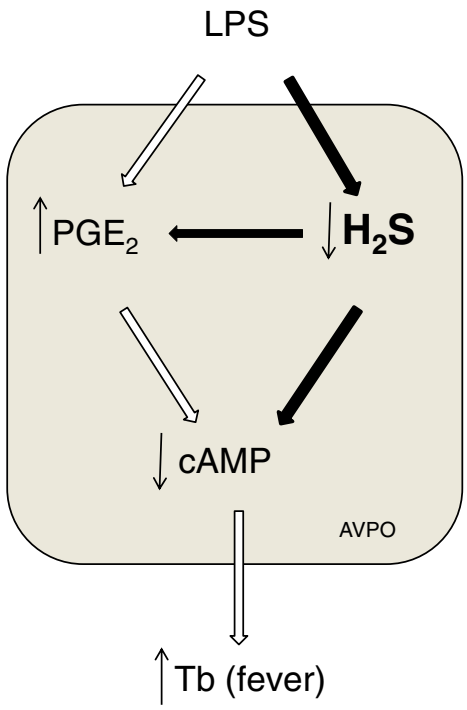

Fig. 7. Possible neurochemical mechanism involved in LPS-induced fever. LPS induces increase in PGE_2 and decrease in H_2S AVPO levels. Increased PGE_2 is known to evoke a decrease in cAMP, ultimately resulting in fever (see text for details). The LPS-induced reduction of H_2S favors the increase in PGE_2 and diminishes the stimulatory effect of the gas on the synthesis of cAMP. These modulatory effects of H_2S on both PGE_2 and cAMP possibly represent the ways by which an increase in the AVPO levels of this gaseous neuromodulator can attenuate fever, thus revealing its potent antipyretic role in the preoptic hypothalamus. Solid arrows represent the novelties of the present study. LPS, lipopolysaccharide; PGE_2, prostaglandin E_2; cAMP, cyclic AMP; Tb, deep body temperature; H_2S, hydrogen sulfide; AVPO, anteroventral preoptic region of the hypothalamus.

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